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Multiproxy diet analysis of the last meal of an early Holocene Yakutian bison

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Abstract

Pollen, botanical macrofossils, chemical components and ancient DNA were studied in samples from the rumen of a frozen Yakutian bison (*Bison priscus*) that lived ca. 10,500 calendar years before present. The dialkyl glycerol ether lipid archaeol (2,3-di-O-phytanyl-*sn*-glycerol) was detected and is interpreted to have been derived from methanogenic Archaea in the rumen. This is the oldest reported occurrence of archaeol attributed to digestive tract Archaea. Remains of shrubs (*Alnus*, *Betula*, *Salix*) and Poaceae indicate that the animal probably lived in a landscape of predominantly dry soils, intermixed with wetlands containing herbaceous plant species as indicated by remains of *Comarum palustre, Caltha palustris, Eriophorum, Sparganium, Menyanthes trifoliata* and *Utricularia*. All recorded taxa still occur in the present day Yakutian tundra vegetation. We discuss the representativeness in space and time of the methods used. Both the botanical microfossil and macrofossil records may be biased because of differences in pollen production and dispersal between species, the food choice of the bison, and the season of death of the animal. Similarities and differences are discussed to highlight pitfalls of the individual analytical techniques. We highlight the power of data integration.

Keywords: Bison, early Holocene, last meal, multiproxy, Yakutia

Introduction

The contents of the digestive tracts of frozen animals are an important source of information about the food choice of the animals and for the reconstruction of the former vegetation of the areas where these animals were living (Ukraintseva 1979, 1993; van Geel *et al*., 2008, 2011a,b; Willerslev *et al.* 2014). In 2011 a complete carcass of a frozen bison (*Bison priscus*; Fig. 1a) was found on the eastern shore of the Chukchalakh Lake in the Yana-Indigirka lowland (72° 17'30''N; 140° 54'05''E) within the paleontological reserve 'Yana Mammoths' in Yakutia (Sakha Republic, Russia). Along the eastern shore of the Chukchalakh Lake there is an unnamed hill (558 m a.s.l.). The Yukagir bison was found in the foothills. The deposits (Late Pleistocene Edoma Formation) of the rolling hill landscape are dissected by the Maksunuokha River and the area is covered by numerous lakes. The bison was radiocarbon dated 9310 ± 45 BP (GrA-53290; horn), and 9295 ± 45 BP (GrA-53292; hair) corresponding to the time interval in calendar years of 10,573-10,424 before 1950 AD (http://www.calpal-online.de/). A first report about this bison was published by Boeskorov *et al*. (2013). The bison was a male of about four years old. The carcass is complete, including snout, ears, tail and genitalia. All organs are fully preserved, including the contents of the digestive tract including the rumen, stomach and intestines. We studied material from the rumen in order to reconstruct the species composition of the bison's last meal and the former vegetation of the area where the bison lived. The rumen contents were subsampled to provide separate aliquots of material for each analytical procedure. We applied a multi-proxy approach, including the analysis of microfossils (pollen, spores), macrofossils (fruits, seeds, vegetative remains), lipids and ancient plant DNA.

Methods

Microfossils and macroremains

The preparation of a subsample for the study of microfossils in Russia (RU) was as follows: After thawing the sample was sieved through a sieve with a mesh of 250 µm to remove large particles. Subsequently the material was treated with 10% hydrochloric acid and 10% potassium hydroxide, and then washed with distilled water and centrifuged. After sieving (meshes of 7 µm), the material on the sieve was put in a tube and glycerin was added. The microfossil analysis was conducted with 400x magnification. The preparation of a subsample for microfossil analysis in the Netherlands (NL) was according to Faegri and Iversen (1989) and Moore *et al*. (1991) and the analysis was worked out with 400x and 1000x magnification. Identifications of microfossils are based on Moore *et al*. (1991), Beug (2004), a pollen reference collection and for fungi, on van Geel and Aptroot (2006). For the macrofossil analysis the RU-sample was examined after washing with water and drying, while the NL-sample was not dried but treated according to Mauquoy and van Geel (2007).

Lipids

Lipids were extracted from freeze-dried, ground rumen contents using the methodology of McCartney *et al*. (2013) to optimise recovery of the dialkyl glycerol ether archaeol (2,3-di-O-phytanyl-*sn*-glycerol) if present. Briefly, 6.92 µg of internal standard, 1,2-di-*O*-*rac*-hexadecyl glycerol (Santa Cruz Biotechnology Inc., CA), was added to each sample before lipid extraction and the total lipid extract was obtained using an extraction procedure modified from Bligh and Dyer (1959). Acid methanolysis was used to cleave polar head groups from archaeol. Silica column chromatography was used to separate the total lipid extract into an apolar fraction and a fraction containing predominantly hydroxyl group-bearing components. For this latter fraction, analytes were derivatised to their respective trimethylsilyl (TMS) ethers by adding 50 µl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS), and 50 µl pyridine to the sample and heating at 70 °C for 1 h. Samples were dissolved in ethyl acetate prior to analysis by gas chromatography/mass spectrometry (GC/MS).

GC/MS was conducted using a Trace 1300 GC coupled to an ISQ MS (Thermo Scientific, Hemel Hempstead, UK), equipped with a non-polar fused silica capillary column (CPSil-5CB, 50 m x 0.32 mm x 0.12 mm, Agilent J&W). The following temperature program was used: initial temperature 40 °C, rising to 130°C at 20°C min $^{-1}$, then rising to 300°C at 4°C min $^{-1}$, holding at 300°C for 25 min. The ion source was maintained at 300 °C and the transfer line at 300 °C. The emission current was set to 50 µA and the electron energy to 70 eV. The analyzer was set to scan *m/z* 50-650 with a scan cycle time of 0.6 s.

To quantify archaeol, a calibration curve was produced by analysing by GC-MS 0.1 µg internal standard (1,2-di-*O*-*rac*-hexadecyl glycerol) together with 0.05 µg, 0.1 µg, 0.2 µg, 0.3 µg and 0.4 µg of arcahaeol standard (1,2-di-O-phytanyl-*sn*-glycerol, Avanti Polar Lipids Inc., AL). As described by McCartney *et al*. (2013), the ratio of the peak area of the archaeol standard (A_x) to the peak area of the internal standard (A_{std}) was plotted against the ratio of the archaeol standard (M_x) to the internal standard (M_{std}) and the resulting regression equation for the slope was:

$$A_x / A_{std} = 1.63\ M_x / M_{std} - 0.308$$

The equation was rearranged as follows to allow the amount of archaeol present in to be calculated:

$$M_x = (A_x / A_{std} + 0.308)/1.63 \times 6.92$$

Ancient DNA

DNA extraction - The outer layer of the samples was carefully removed with a scalpel to prevent contaminants in the extractions. Each sample was ground to fine powder in liquid nitrogen with a mortar and pestle. Ca. 100 mg was used for a CTAB extraction (Doyle and Doyle, 1987). A freshly prepared CTAB buffer (2% CTAB, 2% PVP, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1.42 M NaCl, 2% 2-mercaptoethanol) was added to the ground samples before incubation for 1 hour at 65 °C under agitation. DNA was subsequently extracted using chloroform:isoamyl alcohol (24:1), precipitated with ice-cold isopropyl alcohol and re-suspended in 1×TE buffer. The suspension was then re-precipitated with NH4 acetate and pure ethanol at -20 °C for 30 minutes, washed twice in 76% ethanol 10 mM NH4 acetate and the resulting pellet was air dried and re-suspended in 1×TE buffer. Subsequently, aliquots of each extraction were further purified using Promega PCR purification columns. All extractions were carried out in the special ancient DNA facility of Leiden University following established protocols to avoid contamination (Cooper and Poinar, 2000). Extraction and amplification blanks were included in all steps. No visual amplicons were detected in these blanks. Among the final Ion Torrent reads obtained, however, common contaminants in Ancient DNA studies were detected such as *Solanum lycopersicum*, in addition to other taxa exotic to the study site. These reads were excluded from further analyses.

PCR amplification - Amplifications of the plastid *rbcL* DNA barcoding marker were performed using forward primer Z1aF and reverse primer 19bR (Hofreiter *et al*., 2000). Amplification of the plastid intergenic *trnL-trnF* spacer was performed using the forward primer *g* and reverse primer *h* (Taberlet *et al*., 2007). Primers were labeled for sequencing with IonExpress labels. The PCR was carried out in 25µl reactions containing 1U Phire hot start II DNA polymerase, Phire reaction buffer, 1mM $MgCl_2$, 0.1mg/ml BSA, 1% DMSO, 0.05 mM dNTPs, and 0.4 µM of each primer. Amplifications were performed using a 5 min activation step at 98 °C, followed by 40 cycles at 98 °C for 5 s, 55 °C for 20 s and 72 °C for 60 s, and a concluding step at 72 °C for 5 min.

Ion Torrent sequencing - With the use of Ampure XP beads from Agencourt primer dimer was removed from the PCR products generated. The beads were washed with 150 µl 70% EtOH twice and resuspended in 20 µl TE buffer. Cleaned PCR products were quantified using an Agilent 2100 Bioanalyzer DNA High sensitivity chip. An equimolar pool was prepared of the amplicon libraries at the highest possible concentration. This equimolar pool was diluted according to the calculated template dilution factor to target 10-30% of all positive ISPs. Template preparation and enrichment was carried out with the Ion One Touch 200 Template kit with use of the Ion One Touch System, according to the manufacturers protocol. The quality control of the Ion one touch 200 Ion Sphere Particles was done with the Ion Sphere Quality Control Kit using a Life Qubit 2.0. The Enriched Ion Spheres were prepared for sequencing on a Personal Genome Machine (PGM) with the Ion PGM 200 Sequencing kit as described in the protocol and deposited on an Ion-314-chip (520 cycles per run) in a single loading cycle for one sequencing run.

Data analysis - Reads obtained from Ion Torrent sequencing were trimmed for primers and MID label tags with a custom script. Only reads with a length of at least 100 bp and a mean quality score of Q20 or higher were selected for further analysis. Reads were clustered into Operational Taxonomic Units (OTU's) defined by a

sequence similarity of at least 97% using CD-HIT (Li and Godzik, 2006). Singletons were omitted. Representative consensus sequences of each cluster were blasted against NCBI GenBank data for taxonomic identification up to genus and sometimes even species level.

Results

Microfossils

Table 1 shows the results of the microfossil analysis. Percentages are based on the pollen sum (Σ-pollen), which is the total of recorded pollen grains per sample. Non-pollen microfossils were excluded from the sum but their frequencies are expressed on Σ-pollen. The pollen slide contained transport tissue of *Equisetum* (Type 818; Fig. 2f). Type 817 (Fig. 2j) may represent flattened (dented)-globose bryophyte spores, 16-25 µm in diameter and densely covered with ca 1µm wide appendages. Type 819 (Fig. 2k) is an unidentified microfossil (possibly a parasite egg), 28-38 µm in diameter, with a characteristic opening, in diameter ca 50 to 70% of the size of the whole microfossil.

Macrofossils

The samples contained plant debris, fragmented to a varying degree. Volume estimations of individual categories were not possible. In total 55 ml was examined. The majority of the plant debris consisted of vegetative remains, mainly Poaceae (Fig. 1b,c), Cyperaceae and unidentified herbs, with crushed and splintered branches of shrubs without cortex (25 pieces; length 7-20 mm and diameter of 1-3 mm). In the NL-sample the morphology of such a woody branch was studied in detail and it appeared to be a *Salix* twig (Fig. 1f,g). Also *Salix* epidermis (Fig. 1e) was observed in the sample.

Based on fruits and seeds the following taxa could be identified: Poaceae (5 spikelets and some fruits), *Carex* sp. (4 nuts, one of them in the utricle), *Eriophorum* sp. (2 fruits); *Comarum palustre* (1 seed); *Menyanthes trifoliata* (1 seed fragment), Apiaceae (3 fruits). Epidermis fragments of *Equisetum* were of regular occurrence (Fig. 2a-d). In addition remains of the bryophyte *Calliergon* cf. *giganteum* (Fig. 2i), mammal hairs, a fragment of a bird's feather, ephippia of *Daphnia*, and some quartz grains were observed.

Ancient DNA

Taxa with 97-100% query coverage retrieved encompass genera within the Adoxaceae (*Sambucus*), Asteraceae (*Cirsium, Tragopogon*), Betulaceae (*Betula*), Brassicaceae (*Lepidium*), Cyperaceae (*Eriophorum, Carex*), Lentibulariaceae

(*Utricularia*), Menyanthaceae (*Menyanthes*), Plantaginaceae (*Plantago*), Poaceae (*Agrostis, Anthoxanthum*), Ranunculaceae (*Caltha palustris*), Rosaceae (*Comarum palustre, Potentilla, Rubus*), Salicaceae (*Salix*), Sparganiaceae (*Sparganium*) and Equisetaceae (*Equisetum*). From the total number of 21175 Ion Torrent reads obtained, 466 (less than 4%), 4561 (38,6%), 19 (less than 1%) and 6775 (57,3%) were derived from graminoids (*Agrostis, Anthoxantum, Carex, Eriophorum*), dwarf shrubs (*Betula*, *Salix*), trees and shrubs (*Sambucus*), and non-graminoid herbs (all other genera), respectively.

Lipids

Figure 3 shows the *n*-alkane and *n*-alkanol distributions of the rumen contents. *n*-alkanes range from C_{23} to C_{31} with a strong odd over even predominance, maximising at C_{27}. *n*-alkanols range from C_{22} to C_{28}, maximising at C_{28}. This distribution of *n*-alkanes and *n*-alkanols is consistent with a major input of higher plant organic matter (Dove and Mayes, 1996; Maffei, 1996; Bughalo *et al*., 2004, Killops and Killops, 2005).

Figure 4 is a partial gas chromatogram of the alcohol fraction isolated from the rumen contents. The presence of a suite of 5β-stanol components (C_{27} to C_{29}) confirms that this is digested matter, since these compounds are uniquely formed in the digestive tract by biohydrogenation of unsaturated sterols by digestive tract bacteria (Murtaugh and Bunch, 1967). The predominance of the C_{29} 5β-stanols (stigmastanol and epistigmastanol) and the occurrence of the phytosterol sitosterol is consistent with an herbivorous diet (Leeming *et al*., 1996; Bull *et al*., 2002). This is further supported by the presence of pentacyclic triterpenoids α, β and δ-amyrin and taraxerol (van Bergen *et al*., 1997).

The dialkyl glycerol ether lipid archaeol was detected in the alcohol fraction of the rumen contents at a concentration of 3μg/g dry weight of rumen contents. Archaeol is a ubiquitous component of the cell membrane of Archaea, and in faeces has previously been interpreted to be derived from methanogenic Archaea living in the digestive tract of foregut-fermenting herbivorous animals (Gill *et al*., 2010). The material analysed here comprises rumen contents rather than faeces, but a similar origin is proposed for the archaeol, consistent with the ruminant digestive system of bison. This interpretation is supported by the high concentration of 5β-epistigmastanol (on par with that of 5β-stigmastanol) in the bison rumen contents (Fig. 4). 5β-stanols are known to epimerise at the C-3 hydroxyl group under anaerobic conditions (McCalley *et al*., 1981) and similar relative distributions of the epimer have been observed previously in the faeces of ruminants (Gill *et al*., 2010). Gill *et al*. (2010) reported 1μg/g dry weight archaeol from a 2475 year old ruminant (ovi-caprid) coprolite, but this study extends the record of archaeol derived from rumen microbes by 8000 years and is the first report of archaeol from ancient digestive tract contents.

Discussion

Diet - The record of macrofossils directly reflects the food of the bison: *Salix* (Salicaceae), *Carex* and *Eriophorum* (Cyperaceae), Poaceae, *Comarum palustre* (Rosaceae), *Menyanthes trifoliata* (Menyanthaceae), *Equisetum* (Equisetaceae) and Apiaceae. The moss species *Calliergon* cf. *giganteum* (Amblystegiaceae) may have been ingested by chance. These species are all in line with other diets reconstructed from fossil bison remains (Guthrie, 1990; Jahren *et al.*, 1998; Willerslev *et al.*, 2014).

Poaceae were a major component in the pollen spectra, but a recorded clump of pollen grains of Poaceae means that the pollen spectra can be strongly biased by the food choice of the animal and by the fact that inflorescenses - if still full of not yet released pollen grains - may result in over-representation in pollen spectra. Pollen grains ingested during the growing season will represent mainly the taxa that were flowering when the animal collected his food. Therefore the pollen record of intestinal contents may be strongly biased because only a very short period within the flowering season is represented. The presence of *Alnus* pollen in the NL-sample and its absence in the RU-sample and the presence of Fabaceae pollen in the RU-sample shows that the two samples represent different areas where the bison collected its meal.

Palaeo-environment - The microfossil spectra do not necessarily represent taxa that were actively eaten, as pollen may have arrived as 'pollen rain' on the surface of the grazed vegetation. The presence in relatively low pollen percentages of *Alnus* and *Betula* shows that shrubs were present in a landscape that was predominantly characterized by herbaceous taxa.

Eriophorum, Caltha palustris, Comarum palustre, Utricularia and *Menyanthes trifoliata* formed part of the local vegetation of moist sites in the landscape, while the majority of Poaceae were probably growing on dry soils. Based on the study of ancient DNA we have additional data about plant taxa that played a role in the vegetation where the bison collected its last meal. But like pollen rain, the aDNA data do not necessary represent taxa that were actively grazed. It may be that aDNA comes from pollen grains that were ingested by chance. Chloroplast DNA markers were previously sequenced from pollen by for instance Bennett and Parducci (2006) and Singer *et al.* (2006) showing that pollen can be an excellent source for DNA barcoding. Retrieval of *Angelica* (98% coverage), *Caltha palustris* (98%), *Eriophorum* (97%), *Sparganium* (98%) and *Utricularia* (98%), although supported with short reads only, is likely correct as the present distribution of these taxa is congruent with the area where the bison was found and also indicates the presence of moist sites. Additional ancient DNA studies should shed further light on the putative occurrence of these taxa here during the early Holocene. The discrepance between the

results obtained with both plastid DNA barcoding markers used could be explained by primer bias and the length difference in both amplicons. The *trn*L reads were generally shorter (on average just 55 bp long) than the *rbc*L reads (on average 115 bp long). Around 40% of the *trn*L reads were therefore filtered away prior to clustering and blasting.

The bison was radiocarbon dated around 10,500 calendar years before present, which was ca 1000 years after the climate shift from the Younger Dryas to the Holocene. According to Andreev *et al*. (2011) and Andreev and Tarasov (2013) the Late Glacial/Preboreal transition near the Laptev Sea coast that occurred at about 11.5 cal ka BP, was characterized by a significant increase in birch, shrub alder, and willow pollen and the interval from ca. 10.5 to ca. 8.8 cal ka BP was the warmest postglacial episode (the Holocene climatic optimum) in the arctic regions of northern Asia, with an average annual temperature 4^0 C higher than today. This is line with the fact that we retrieved both herbaceous and woody samples from the bison's rumen. Our results are congruent with Willerslev *et al.* (2014) who sequenced other herbivore dung from Siberia from this time period in terms of numbers of reads found as non-graminoid herbs were also the dominating component of the dung analyzed in this study.

Season of death - The excellent preservation of this Yukagir bison may mean that he died and was covered with mud in the warm season (in winter there could have been no solifluction as all soil material was frozen). After coverage with mud the permafrost kept the animal frozen, as only the upper sediment layers thawed during warm seasons.

Added value of multiproxy approach - When interpreting intestinal samples there are pitfalls when using only one individual analytical technique. There are similarities and differences between the various methods used to identify the palaeovegetation and the meal of the bison. Pollen analysis gives an overview of taxa present in the landscape, but the pollen rain, falling on the plants before they were ingested, does not necessarily reflect the vegetation coverage and the taxa in the ingested food. There is a general bias because of underrepresentation of insect-pollinated taxa and the over-representation of wind-pollinated taxa. Pollen spectra may also be strongly biased because of the food choice of the animal and the flowering period of the various species in the landscape in relation to the days of the last meal. Ingestion of inflorescences may cause a strong over-representation of the flowering taxon. Our combined results show that a total of 24 plant and three fungal families occurred in the palaeo-environment. Of these families, ca. 60% are wind-pollinated and ca. 40% insect-pollinated. This means that there is indeed a bias towards wind-pollinated plants. Of the 24 plant families found, a total of 16 families were discovered with pollen analysis (of which 7 families were only found using this method), a total of 16 were retrieved with Ancient DNA analysis (of which 6 families were not detected by the other methods), and a total of 7 families by analysis of the

macroremains (of which one family was not picked up by the other methods). Three families (Apiaceae, Cyperaceae and Poaceae) were retrieved by all methods. Pollen analysis retrieved the highest number of unique families. The combined results, however, can be interpreted as a respresentative reflection of the species composition of the last meal of the animal.

The inclusion of lipid analysis in the multi-proxy study confirms an herbivorous diet, but also provides a unique insight into the digestive physiology of an ancient animal. The presence of specific lipids in the rumen content is indicative of particular groups of digestive tract microbes, for example 5β-stanols (indirectly) indicate the presence of digestive tract bacteria and archaeol is interpreted to have been derived from methanogenic Archaea.

Conclusions

Our study of the remains found in the rumen of this bison indicate that it lived on relatively dry soils, intermixed with wetlands and that both herbaceous and woody species were present in its palaeo-environment. This result is in line with earlier studies on the diet of fossil bison, the area and period studied. The identifications retrieved give an impression of the vegetation types in the landscape where the animal collected its food, but a selective diet and habitat choice and the very short part of the flowering season represented in this sample may have given a strong bias to the botanical record. We therefore hope that more fossils from the same area will become available to supplement this study. Similar to Jørgensen et al. (2012) we advocate an integrated approach for studying these fossils, as we show that separate methods are of limited value as compared to combined palynological, macrobotanical, chemical and molecular studies.

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Captions:

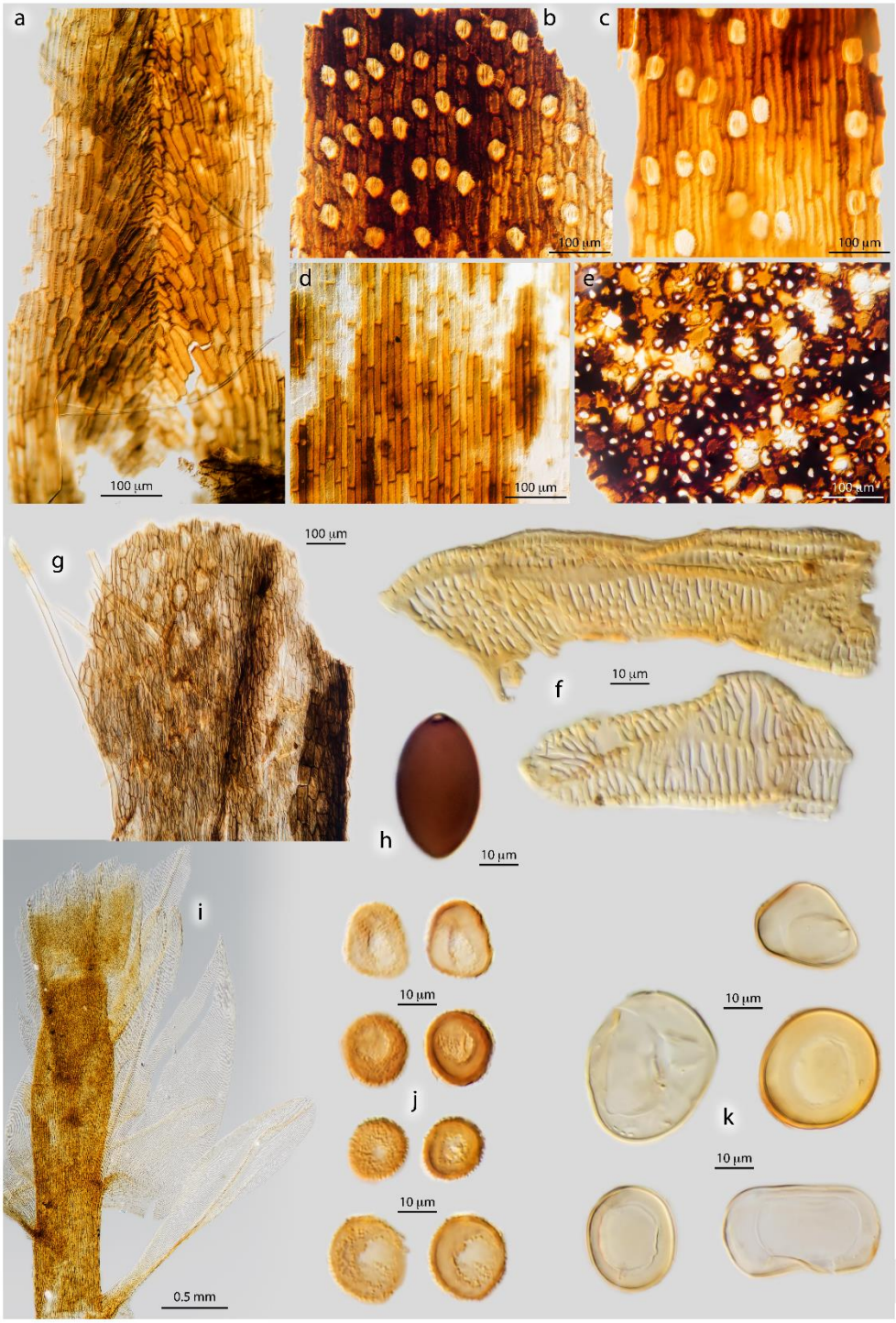
Figure 1: The Yukagir bison (1a) and plant remains from the rumen. 1b,c: epidermis of Poaceae. 1d: monocot leaf margin. 1e: *Salix* epidermis. 1f,g: *Salix* wood fragments.

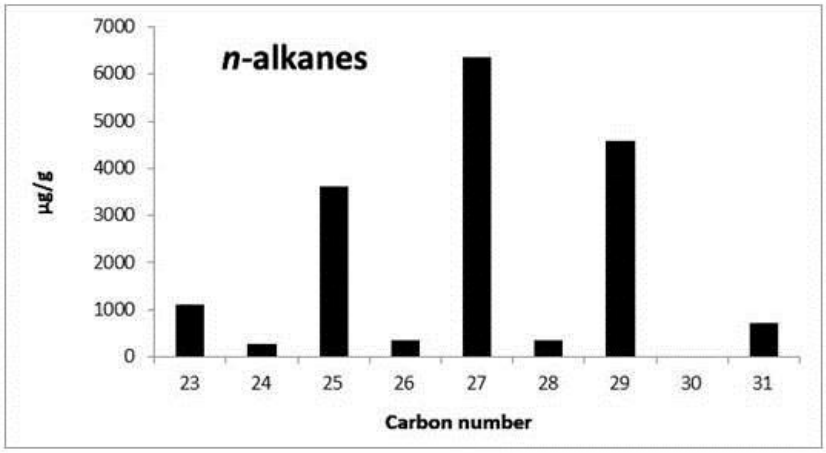
Figure 2: Plant remains and some unidentified microfossils from the rumen of the Yukagir bison. 2a: leaf of *Equisetum*. 2b,c: *Equisetum* epidermis with stomata. 2d: *Equisetum* epidermis. 2e: part of *Equisetum* diaphragm. 2f: *Equisetum* transport tissue (Type 818). 2g: unidentified root fragment with hairs. 2h: ascospore of *Sordaria* type. 2i: *Calliergon* cf. *giganteum*. 2j: unidentified bryophyte spores (Type 817). 2k: unidentified microfossils (Type 819: parasite eggs?).

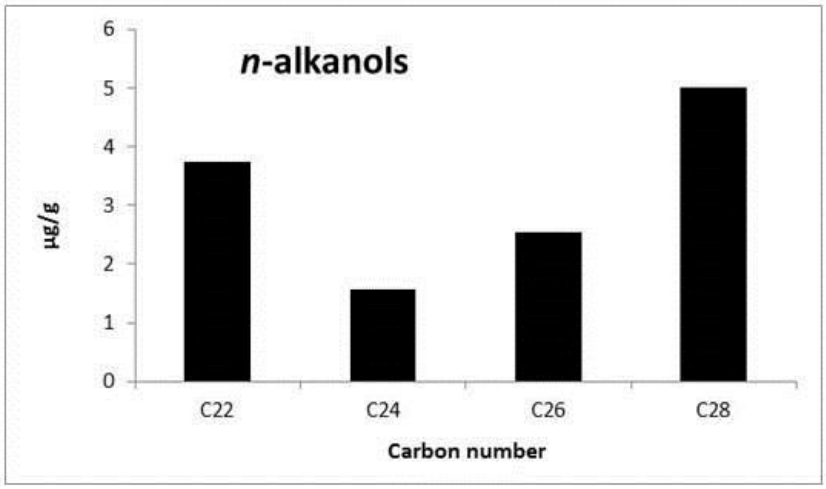
Figure 3: n-alkyl lipids from bison rumen contents

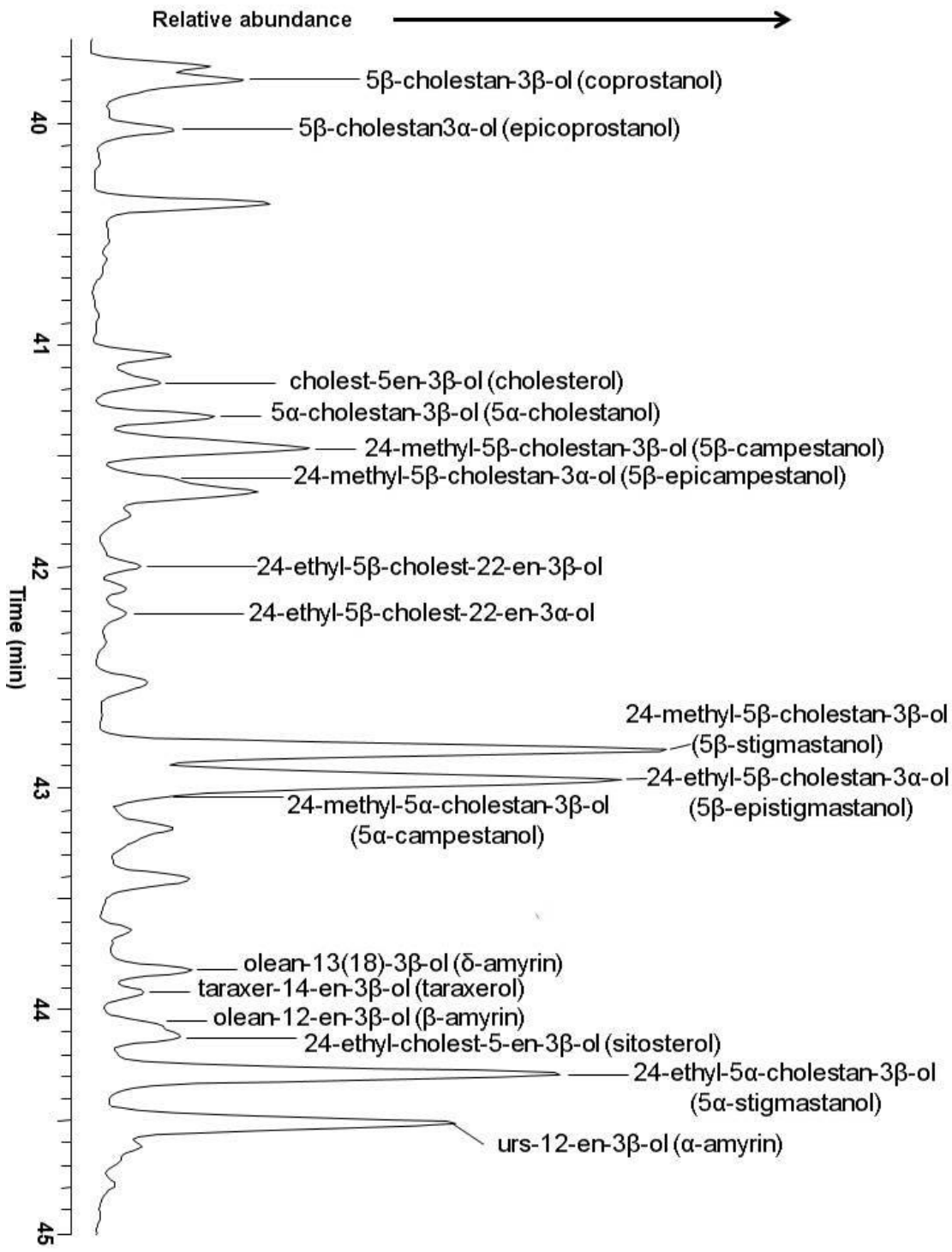
Figure 4: Partial gas chromatogram of the alcohol fraction isolated from the bison rumen contents. Trivial names are given in brackets.

Table 1: Microfossil spectra, macrofossil and Ancient DNA data. NL: analysis by BvG; RU: analysis by NR, ST and SZ. Non-pollen palynomorphs were recorded in the NL sample only. Observations that were made after finishing the counting procedure have been indicated with +.


a
b
c
100 µm
100 µm
100 µm
d
e
100 µm
100 µm
g
100 µm
f
10 µm
h
10 µm
i
10 µm
10 µm
j
k
10 µm
10 µm
0.5 mm


n-alkanes
µg/g
7000
6000
5000
4000
3000
2000
1000
0
23
24
25
26
27
28
29
30
31
Carbon number


n-alkanols
µg/g
6
5
4
3
2
1
0
C22
C24
C26
C28
Carbon number


Relative abundance
Time (min)
40
41
42
43
44
45
5β-cholestan-3β-ol (coprostanol)
5β-cholestan3α-ol (epicoprostanol)
cholest-5en-3β-ol (cholesterol)
5α-cholestan-3β-ol (5α-cholestanol)
24-methyl-5β-cholestan-3β-ol (5β-campestanol)
24-methyl-5β-cholestan-3α-ol (5β-epicampestanol)
24-ethyl-5β-cholest-22-en-3β-ol
24-ethyl-5β-cholest-22-en-3α-ol
24-methyl-5β-cholestan-3β-ol
(5β-stigmastanol)
24-ethyl-5β-cholestan-3α-ol
(5β-epistigmastanol)
24-methyl-5α-cholestan-3β-ol
(5α-campestanol)
olean-13(18)-3β-ol (δ-amyrin)
taraxer-14-en-3β-ol (taraxerol)
olean-12-en-3β-ol (β-amyrin)
24-ethyl-cholest-5-en-3β-ol (sitosterol)
24-ethyl-5α-cholestan-3β-ol
(5α-stigmastanol)
urs-12-en-3β-ol (α-amyrin)


a
b
50 µm
c
50 µm
d
50 µm
e
50 µm
f
50 µm
g
50 µm

Table 1. Microfossil spectra, macrofossil and Ancient DNA data. NL: analysis by BvG; RU: analysis by NAR, SST and SVZ. Non-pollen palynomorphs were recorded in the NL sample only. Observations that were made after finishing the counting procedure have been indicated with +.

Family	**Genus/species**	**Pollensum (NL) 406 %**	**Pollensum (RU) 246 %**	**DNA barcoding marker (nr of reads)**	**Most similar GenBank accession**	**Query cover (bp)**	**E value**	**Macroremains vegetative**	**Macroremains fruits/seeds**
Phanerogams:									
Adoxaceae	*Sambucus* sp.			*rbcL* (19)	KC584884	129	4e-60		
Apiaceae	undiff.	13.2	4.5						+
	Angelica sp.			*rbcL* (59)	GQ248549	131	1e-55		
	Crithmum maritimum			*rbcL* (30)	JN891085	129	4e-60		
	Daucus sp.			*rbcL* (2061)	DQ898156	137	8e-63		
	Heracleum sp.			*rbcL* (141)	KF613112	130	2e-59		
	Peucedanum sp.			rbcL (4292)	KC295127	129	4e-60		
Asteraceae	*Artemisia* sp.	0.2							
	Cirsium sp.			*rbc*L (331)	KC589830	128	2e-59		
	Tragopogon sp.			*rbcL* (881)	JX848433	130	3e-62		

		Tubuliflorae	0.2							
Betulaceae		*Alnus* sp.	6.1							
		Betula sp.	2.0		0.8	*rbcL* (64)	FJ423687	137	8e-63	
		Betula sect. *Nanae*			4.5					
		Betula sect. *Albae*			1.6					
Brassicaceae		*Lepidium* sp.				*rbcL* (30)	JX848442	138	2e-59	
Caryophyllaceae			+							
Cyperaceae		undiff.	5.6		6.5				+	+
		Carex sp.				*trnL* (70)	JX644766	107	8e-42	+
		Eriophorum sp.				*trnL* (388)	JN873698	105	3e-45	+
Fabaceae		undiff.			2.8					
Lentibulariaceae		*Utricularia* sp.				*rbcL* (15)	KC997777	137	4e-61	
Liliaceae		undiff.			0.4					
Menyanthaceae		*Menyanthes trifoliata*				*rbc*L(1024)	JN965669	127	6e-59	+
Pinaceae	undiff.			0.4						
		Pinus subgenus								

	Diploxylon		0.4						
	Pinus subgenus								
	Haploxylon		0.4						
Plantaginaceae	*Plantago* sp.			rbcL (57)	FM207430	137	2e-59		
Poaceae	undiff.	70.7	72.0					+	+
	Agrostis sp.			*rbcL* (4)	JX848486	129	10e-57		
	Anthoxanthum sp.			*rbcL* (6)	JX848487	135	5e-60		
Polygonaceae	*Rumex* sp.			*rbcL* (14)	JN234940	138	4e-61		
Ranunculaceae	undiff.		0.4						
	Caltha palustris			*rbcL* (691)	JN890942	129	2e-58		
Rosaceae	*Comarum palustre*			*rbcL* (1253)	JN893736	121	2e-53		+
	Potentilla sp.	1.2		*rbcL* (32)	JX848521	136	6e-59		
	Rubus sp.			*rbcL* (45)	JX848533	136	1e-60		
Salicaceae	*Salix* sp.	0.7	0.4	*rbcL* (4497)	KC483930	102	4e-45	+	
Sparganiaceae	*Sparganium* sp.			*rbcL* (24)	KC484142	127	1e-55		

Cryptogams:

Amblystegiaceae	*Calliergon* cf. *giganteum*							+	
Bryophyta	Type HdV-817 (bryophyte spores)	74.8							
Dennstaedtiaceae	*Pteridium* sp.	0.4							
Equisetaceae	*Equisetum* sp.	5.6	0.4	*rbcL* (87)	JN968380	137	2e-59		+
	Type HdV-818 (*Equisetum* transp. tissue)	18.8							
Indet	Type HdV-819	7.6							
Polypodiophyta	undiff.		4.5						
Sphagnaceae	*Sphagnum* sp.		0.8						
Zygnemataceae	*Spirogyra*	+							
Fungi:									
Magnaporthaceae	*Clasterosporium caricinum*	0.7							

	(T.HdV-126)	
Sporormiaceae	*Sporormiella*-type (T.HdV-113)	0.2
Sordariaceae	*Sordaria*-type (T.HdV-55A)	+